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Activities of Native and Tyrosine-69 Mutant Phospholipases A₂ on Phospholipid Analogues. A Reevaluation of the Minimal Substrate Requirements[†]

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ABSTRACT: The role of Tyr-69 of porcine pancreatic phospholipase A₂ in substrate binding was studied with the help of proteins modified by site-directed mutagenesis and phospholipid analogues with a changed head-group geometry. Two mutants were used containing Phe and Lys, respectively, at position 69. Modifications in the phospholipids included introduction of a sulfur at the phosphorus (thionophospholipids), removal of the negative charge at phosphorus (phosphatidic acid dimethyl ester), and reduction (phosphonolipids) or extension (diacylbutanetriol choline phosphate) of the distance between the phosphorus and the acyl ester bond. Replacement of Tyr-69 by Lys reduces enzymatic activity, but the mutant enzyme retains both the stereospecificity and positional specificity of native phospholipase A₂. The Phe-69 mutant not only hydrolyzes the R_p isomer of thionophospholipids more efficiently than the wild-type enzyme, but the S_p thiono isomer is hydrolyzed too, although at a low (~4%) rate. Phosphonolipids are hydrolyzed by native phospholipase A₂ about 7 times more slowly than natural phospholipids, with retention of positional specificity and a (partial) loss of stereospecificity. The dimethyl ester of phosphatidic acid is degraded efficiently in a calcium-dependent and positional-specific way by native phospholipase A₂ and by the mutants, indicating that a negative charge at phosphorus is not an absolute substrate requirement. The activities on the phosphatidic acid dimethyl ester of native enzyme and the Lys-69 mutant are lower than those on the corresponding lecithin, in contrast to the Phe-69 mutant, which has equal activities on both substrates. Our data suggest that in porcine pancreatic phospholipase A₂ fixation of the phosphate group is achieved both by an interaction with the phenolic OH of Tyr-69 and by an interaction with the calcium ion. In the mutant Y69K the ε-NH₂ group can play a role similar to that of the Tyr OH group in native PLA₂. The smaller side chain of the Y69F mutant can interact with more bulky head groups, allowing for relatively high enzymatic activities on modified phospholipids. On the basis of these results, a reevaluation of the minimal substrate requirements of phospholipase A₂ is presented.

The lipolytic enzyme phospholipase A₂ (PLA₂) specifically cleaves the 2-acyl ester bond of phosphoglycerides in a calcium-dependent reaction. The mechanism by which the calcium ion activates this enzyme is in debate. For some phospholipases from snake venoms it has been suggested that the enzyme binds calcium ions and substrate in an ordered way [for a review, see Verheij et al. (1981a)]. For PLA₂s isolated from mammalian pancreatic tissue the situation is more complex. At pH values of 6 or lower, all pancreatic phospholipases readily bind to micelles of substrates or substrate analogues in the absence of calcium ions, although no hydrolysis occurs as long as no calcium ion is bound in the active site. Above pH 6 the binding of some PLA₂s to lipid aggregates becomes calcium dependent (van Dam-Mieras et al., 1975). Comparison of naturally occurring isozymes as well as site-specific mutagenesis studies revealed that two aspartate residues, i.e., Asp-66 and Asp-71, are involved in the binding of a second calcium ion (Donné-Op den Kelder et al., 1983; van den Bergh et al., 1989a). Hence, it was assumed that the

role of this second calcium ion is to improve the binding of PLA₂ to micelles and thereby to increase the turnover number of the enzyme (van den Bergh et al., 1989a).

Chemical modification studies as well as X-ray analyses have shown that the "catalytic" calcium ion is bound to Asp-49 in the vicinity of the active site histidine-48 (Fleer et al., 1981; Dijkstra et al., 1983). Although several di- and trivalent cations bind stoichiometrically to PLA₂s from pancreatic tissue or snake venom, the enzymatic activity in these cases is only a few percent at the most. These observations suggested (Verheij et al., 1980) that the calcium ion in the active site serves two functions: the fixation of the phosphate of the polar head group and the polarization of the carbonyl of the susceptible ester bond. An X-ray structure of the ternary complex between phospholipase A₂, the Ca²⁺ ion, and a substrate analogue molecule could elucidate the relative position of these molecules. Unfortunately, the crystallization of such a complex has not yet been achieved. There is, however, experimental evidence accumulating that sheds more light on these interactions. The fixation of the phosphate by the Ca²⁺ ion was merely suggested by intuition, until Tsai et al. (1985) showed that the Ca²⁺ ion is probably in contact with the *pro-R* oxygen

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of this phosphate. More recently, Kuipers et al. (1989a) suggested that the hydroxyl of Tyr-69 is also in contact with the phosphate function and that this interaction contributes to the stereospecificity of phospholipase A₂. We decided to study these interactions in more detail by making use of native porcine pancreatic PLA₂ and two mutants in which Tyr-69 was replaced by a Phe (no hydrogen-bond donor) and a Lys (a hydrogen-bond donor), respectively, and substrate molecules in which the polar head group was changed. Thus, molecules lacking the negative charge on phosphorus and lecithin analogues with an increased or decreased distance between the phosphorus and the susceptible ester bond were included. The results are discussed in terms of the proposed catalytic mechanism (Verheij et al., 1980) and the minimal substrate requirements of phospholipase A₂ (de Haas et al., 1968).

MATERIALS AND METHODS

Construction of Mutant Phospholipases. Mutant Y69K¹ was constructed essentially as described for mutant Y69F (Kuipers et al., 1989a). Briefly, the mutagenic oligonucleotide 5'-GCT TTC GGT C*TT* GGG ATT G-3' was used in the gapped duplex procedure, for which amber selection was employed (Kramer et al., 1984). Asterisks denote the places of base mutations in the mutagenic primer, relative to the wild-type sequence. The cDNA encoding the mutant PLA₂ species was sequenced, and a *Bst*X1-*Bgl*II fragment containing the mutation was ligated into the expression vector pOK13. After transformation and expression in *Escherichia coli* K-12 strain MC4100 (Silhavy et al., 1984), containing plasmid pCI857, the mutant PLA₂ was obtained by tryptic cleavage of reoxidized fusion protein (de Geus et al., 1987). Purification was achieved by CM-cellulose chromatography at pH 5 and 6, followed by chromatography on DEAE-cellulose at pH 8.

Phospholipids. The 1,2-diacyl-*sn*-glycerols used in this study were prepared by acylation of 3-*O*-benzyl-*sn*-glycerol (Bonsen et al., 1972a) followed by catalytic hydrogenolysis. Phosphorylation with an excess of phosphorusoxy-trichloride and subsequent reaction with methanol yielded the dimethyl ester phosphatidic acid, which was purified by silicic acid chromatography with hexane-ether mixtures as eluants. The 1,2-*sn*-diacyllecithin was prepared from the diglyceride by phosphorylation with 2-chloro-2-oxo-1,3,2-dioxaphospholane followed by ring opening with trimethylamine essentially as described by Chandrakumar and Hajdu (1983). The thionolecithin 1,2-didodecanoyl-*sn*-glycero-3-thionophosphocholine was synthesized from the corresponding diglyceride as described by Nifant'ev et al. (1978). The *R_p* and *S_p* isomers were prepared from the mixture by degradation with phospholipase A₂ from *Crotalus adamanteus* venom, separation of the products, and reacylation of the lyso *R_p* derivative, essentially as described by Bruzik et al. (1983).

The synthesis of *rac*-1-tetradecanoyl-2-dodecanoyl-phosphonolecithin was carried out, starting from the barium salt of *rac*-3-deoxyglycerol 3-phosphonate (Baer & Basu, 1969). After conversion of the barium salt to the free acid with sulfuric acid, the starting compound was converted into the dibenzyl ester with phenyldiazomethane. This compound was monoacylated in dry toluene at 0 °C with 1 equiv of tetradecanoyl chloride in the presence of pyridine. The reaction mixture was purified by silicic acid column chromatography with hexane-diethyl ether mixtures as eluants. Pure *rac*-1-tetradecanoyl-3-deoxyglycerol 3-phosphonate dibenzyl

ester (*R_f* 0.35 in CHCl₃/methanol = 97:3) was obtained, free from traces of the corresponding 2-acyl derivative (*R_f* 0.31). The 1-acyl compound was acylated with a small excess of dodecanoyl chloride. The triester was converted to the acid by catalytic hydrogenolysis with palladium as catalyst, and this compound was allowed to react with choline tosylate in trichloroacetonitrile and pyridine as solvent (Rosenthal, 1966). The final product was purified by isocratic silicic acid chromatography with CHCl₃/methanol/water = 65/15/2 as solvent. The purity and identity of the product and the intermediates were verified by ¹H NMR. The racemic phosphonolecithin was separated into the *sn*-1 and *sn*-3 isomers with the aid of phospholipase A₂ essentially as was described for the thionolecithin (Bruzik et al., 1983). The steps include a short incubation until about 40% hydrolysis was reached, followed by separation of the lysolecithin and the diacyllecithin by silicic acid chromatography. The lyso compound was reacylated by standard procedures to yield 1-tetradecanoyl-2-dodecanoyl-*sn*-3-phosphonolecithin. The diacyllecithin that was recovered after the first phospholipase incubation contained the *sn*-1 isomer, contaminated with the *sn*-3 isomer. A second prolonged phospholipase A₂ digestion removed the contaminating *sn*-3 isomer, and the pure *sn*-1 isomer was isolated by silicic acid chromatography.

Phospholipase Assays. The activities of phospholipases were determined quantitatively in a titrimetric assay at pH 8 in the presence of 1 mM borate, 25 mM CaCl₂, and 100 mM NaCl at 25 °C with a 10 mM sodium hydroxide solution. The solution was flushed with nitrogen prior to the addition of substrate, and during the experiment the reaction vessel was kept under a nitrogen atmosphere. Lecithins and analogues were solubilized with sodium taurodeoxycholate (2 mol/mol of phospholipid) to form mixed micelles. The phosphorus triester was solubilized with the aid of either deoxycholate, taurodeoxycholate, or choline *n*-hexadecyl phosphate. Activities on monomeric *rac*-1,2-dihexanoyldithiolecithin were determined at pH 8 in the presence of 200 mM Tris, 100 mM NaCl, and 100 mM CaCl₂, as described previously by Volwerk et al. (1979).

Direct Binding of PLA₂ to Monomers and Micelles of Phospholipids. The affinity of phospholipase A₂ for monomers and micelles was determined by following the increase of tryptophan fluorescence or by ultraviolet difference spectroscopy upon addition of increasing concentrations of the nonhydrolyzable substrate analogues choline *n*-dodecyl phosphate (cmc 1.3 mM) for monomer binding and choline *n*-hexadecyl phosphate (cmc 10 μM) for micelle binding. Assays were performed in a buffer containing 100 mM NaOAc, 50 mM CaCl₂, and 100 mM NaCl at pH 6.0. From saturation curves, obtained with lipid monomers, a *K_d* value can directly be derived. The data concerning micelle binding were analyzed in terms of the binding of the enzyme to a theoretical lipid particle consisting of *N* monomers with a dissociation constant *K_d*. The *NK_d* value, discussed extensively by de Araujo et al. (1979), is the experimental concentration at which 50% of the enzyme is saturated with micelles. Experimental conditions were as indicated before (van Dam-Mieras et al., 1975), except that in the fluorescence experiments the excitation wavelength was 280 nm. The binding of PLA₂ to micelles of choline *n*-hexadecyl phosphate (C16PN) was determined also by gel filtration on Sephadex G-75 columns as described before (de Araujo et al., 1979).

NMR Studies. ³¹P NMR spectra were recorded at 81.015 MHz on a Bruker WP-200 WB spectrometer equipped with a multinuclear 10-mm probe. To samples of 2.5 mL containing

¹ Abbreviations: C12PN, choline *n*-dodecyl phosphate; C16PN, choline *n*-hexadecyl phosphate; cmc, critical micellar concentration; F, phenylalanine; K, lysine; Y, tyrosine.

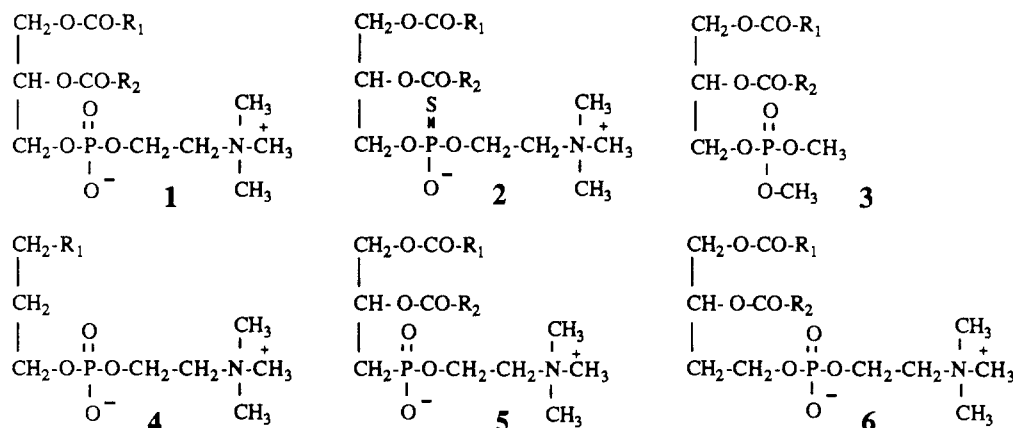


FIGURE 1: Schematic structures of several phospholipid analogues. R_1 and R_2 represent alkyl chains of variable lengths. (1) 1,2-diacyl-*sn*-glycero-3-phosphocholine (lecithin); (2) 1,2-diacyl-*sn*-glycero-3-thionophosphocholine (thionolecithin); (3) 1,2-diacyl-*sn*-glycerol 3-(dimethyl phosphate); (4) choline *n*-alkyl phosphate; (5) choline 1,2-bis(acyloxy)-3-propyl-phosphonate (phosphono analogue of lecithin); (6) 1,2-diacyl-1,2,4-butanetriolcholine phosphate.

Table I: Enzymatic Activities and Binding of Wild-Type and Two Mutant Phospholipases A_2 Using Monomeric and Micellar Substances and Substrate Analogues^a

enzyme	diC6dithioPC	C12PN	diC8PC		C16PN
	k_{cat}/K_m ($s^{-1} \cdot M^{-1}$)	K_d (mM)	V_{max} ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)	K_m^{app} (mM)	NK_d (μM)
wild type	900	0.3	2000	3.7	380
Lys-69	1350	0.3	230	10.6	nd ^b
Phe-69	1300	0.3	440	9.5	480

^a Monomeric substrates (analogues): diC6dithioPC, *rac*-1,2-dihexanoyldithiolecithin; C12PN, choline *n*-dodecyl phosphate; Micellar substrates (analogues): diC8PC, 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine; C16PN, choline *n*-hexadecyl phosphate. Standard errors did not exceed 10% in each case. For details, see Materials and Methods.

^b Was not possible to determine; see text.

a 5 mM solution of R_p or S_p isomer was added 10% D_2O for deuterium lock. Chemical shifts are referenced to external 85% H_3PO_4 (0 ppm), a positive signal indicating a downfield shift. Spectra were recorded at 293 K with a sweep width of 2000 Hz with 8K data points, usually 128 transients with 90° pulses, and a cycle time of 2 s. Broad-band proton noise decoupling was employed. Prior to Fourier transformation the data were multiplied by a Gaussian window function (LB-2, GB 0.2).

RESULTS

Enzymatic Activities of Tyr-69 Mutant Phospholipases. In a previous study (Kuipers et al., 1989a) it was shown that replacement of tyrosine-69 by phenylalanine results in an active enzyme with a changed stereospecificity. This substitution removed a possible hydrogen bridge contact, and hence, the substitution of lysine for tyrosine-69 was also of interest. In Table I the activities of this mutant phospholipase are given, and for reasons of comparison the data of native and Y69F PLA₂s are also included. It is clear that the Y69K mutant PLA₂ has rather low activity on aggregated substrates as compared to native and Y69F PLA₂s. This was somewhat unexpected, since a lysyl residue is often encountered at this position in many venom PLA₂s. When monomeric substrates are used, all three PLA₂s show comparable k_{cat}/K_m values.

Direct Binding Studies. The addition of choline *n*-alkyl phosphates (Figure 1, analogue 4) to native porcine pancreatic phospholipase A_2 causes spectral perturbations, which can be visualized by ultraviolet difference spectroscopy. The addition of these product analogues at concentrations below the cmc induces a perturbation spectrum that is characteristic of (a) perturbed tyrosine residue(s). Above the cmc in the presence

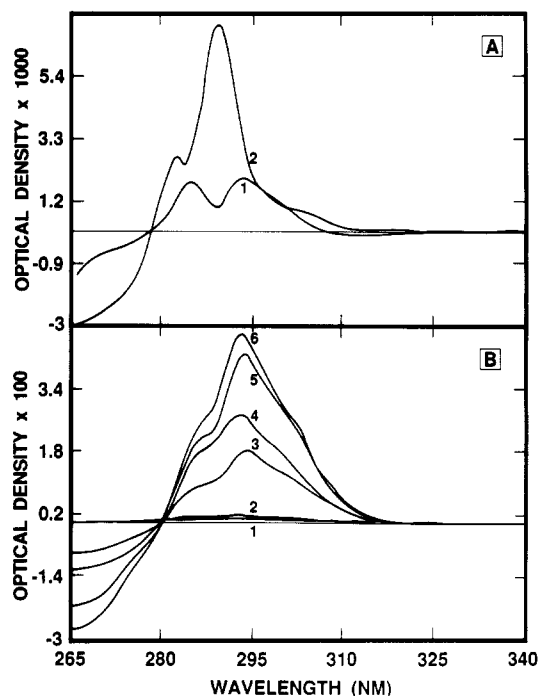


FIGURE 2: (Panel A) UV difference spectroscopy spectra obtained upon binding of wild-type and Y69F PLA₂s to the monomeric product analogue choline *n*-dodecyl phosphate: (curve 1) Y69F PLA₂ (27.7 μM) with 0.7 mM C12PN; (curve 2) wild-type PLA₂ (31.3 μM) with 0.7 mM C12PN. (Panel B) UV difference spectroscopy spectra obtained upon binding of wild-type, Y69K, and Y69F PLA₂s to the micellar product analogue choline *n*-hexadecyl phosphate: (curve 1) Y69K PLA₂ (27.4 μM) with 0.57 mM C16PN; (curve 2) Y69F PLA₂ (27.4 μM) with 2.54 mM C16PN; (curve 3) Y69F PLA₂ (34.3 μM) with 0.57 mM C16PN; (curve 4) wild-type PLA₂ (34.2 μM) with 0.57 mM C16PN; (curve 5) Y69F PLA₂ (34.3 μM) with 2.54 mM C16PN; (curve 6) wild-type PLA₂ (34.2 μM) with 2.54 mM C16PN. For details of the experiments, see Materials and Methods.

of micelles not only is this tyrosine perturbation observed but also a perturbation of the single tryptophan-3 of PLA₂. It has been suggested by Meijer et al. (1979) that Tyr-69 causes the spectral changes after addition of monomeric substrate analogues, and it was therefore of interest to analyze the spectra of Y69F and Y69K after addition of choline alkyl phosphates at concentrations both below and above the cmc. As can be seen from Figure 2A the addition of monomeric concentrations of choline *n*-dodecyl phosphate (C12PN) to native PLA₂ induces a tyrosine perturbation. From a series of such spectra at varying lipid concentrations a dissociation constant of 0.3

mM was calculated, in agreement with previously reported values (Meijer et al., 1979). In contrast, no such spectral changes were observed after addition of this detergent to both mutant phospholipases. The Y69F mutant PLA₂ lacked the tyrosine perturbation peak at 289 nm, and instead, two smaller peaks were visible (Figure 2A). These peaks could be due to a weak tryptophan perturbation, in agreement with the fact that tryptophan fluorescence increases slightly upon addition of choline *n*-dodecyl phosphate (data not shown). From the increase of the fluorescence signal, dissociation constants of 0.3 mM were obtained both for native PLA₂ and for Y69F PLA₂. Thus Tyr-69 seems indeed to be one of the chromophores involved in the interaction of monomeric substrate (analogues) with native porcine pancreatic phospholipase A₂. When the titrations were carried out with the Y69K mutant, signals were observed neither with ultraviolet nor with fluorescence spectroscopy. This could mean that this mutant PLA₂ does not bind monomeric choline alkyl phosphates, but in view of the kinetic data of Y69K PLA₂ with monomeric substrates we consider this possibility unlikely, and we suppose that the lack of signal is due to intramolecular quenching.

The addition of the product analogue choline *n*-hexadecyl phosphate at concentrations above its cmc induced spectral changes in native and the Y69F mutant that were dominated by an absorption at 294 nm (Figure 2B). Such a spectrum is characteristic of a tryptophan perturbation as has been concluded before to be the case for native PLA₂ (van Dam-Mieras et al., 1975). From the spectral changes the dissociation constants (NK_d) of native and Y69F PLA₂ were calculated to be 380 and 480 μ M, respectively (Table I). The fact that these values are similar to the binding constants for monomeric substrate analogues is just a coincidence. The shape of the spectra (Figure 2B) and the gel filtration studies that were carried out (see below) indicate that we are dealing with the formation of a lipid-enzyme complex of high aggregation number. Small signals were observed when the titration was carried out with the Y69K mutant. To test whether this mutant still binds to micelles, we carried out direct binding experiments on Sephadex G-75 columns (see Materials and Methods). On such columns, PLA₂ bound to the C16PN micelles (molecular mass \sim 70 kDa) will coelute near the void volume of the column, in contrast to unbound PLA₂, which will elute at a position corresponding to a molecular mass of 14 kDa. When we carried out this experiment, the mutant Y69K comigrated with choline *n*-hexadecyl phosphate micelles in the void volume of Sephadex G-75 (data not shown). We therefore conclude that the low signal in the spectroscopic experiment cannot be due to a lack of binding.

Activities on Thionolecithins. The *R_p* isomer of thionolecithins (Figure 1, analogue 2) has been shown to be a substrate for PLA₂s from various sources (Tsai et al., 1985). The activities of native and of mutant PLA₂s were determined on 1,2-didodecanoylleicithin (Figure 1, analogue 1) and on its *R_p* and *S_p* thionoanalogues. It is known that the *R_p* and *S_p* isomers of dipalmitoylthionolecithin have different phase transitions (Chang et al., 1986). Therefore, we tested the medium-chain phospholipids in the presence of taurodeoxycholate in a 2-fold excess to obtain clear mixed micellar solutions. Such micelles are to be preferred over the use of bilayer structures where the activity of phospholipases is strongly influenced by physicochemical parameters (Jain & Berg, 1989). From the results presented in Table II it can be seen that, with the normal phospholipid as a substrate, the native enzyme is more active than the Phe and Lys mutants. The ratios between the activities are, however, comparable to the

Table II: Activities of Wild-Type and Mutant PLA₂s on Normal Lecithins and on the Two Isomers of Thionolecithins^a

enzyme	activity (μ mol·min ⁻¹ ·mg ⁻¹)		
	diC12PC	diC12thionoPC	
		<i>R_p</i> isomer	<i>S_p</i> isomer
wild type	55	13	<0.1
Lys-69	11	2	<0.02
Phe-69	14	30	1.2

^a diC12PC, 1,2-didodecanoyl-*sn*-glycero-3-phosphocholine; diC12thionoPC, 1,2-didodecanoyl-*sn*-glycero-3-thionophosphocholine. Kinetic assays were performed with a pH-stat, as described under Materials and Methods. Standard errors did not exceed 10% of the reported value.

ratios with dioctanoylleicithin as a substrate, demonstrating that the presence of detergents does not give rise to conflicting results. The activity of the native PLA₂ on the *R_p* thionolecithin is about 4 times lower than that on the normal lecithin. More striking is the observation that the two mutant PLA₂s respond quite differently to the introduction of a sulfur in the phosphate moiety. The Y69K mutant has a reduced activity; the Y69F mutant has an increased activity on the thiono analogue compared to regular lecithin. Also, with *S_p* isomer there is a clear distinction: both native PLA₂ and the Y69K mutant show very low activities on this compound, whereas the Y69F mutant has about 4% activity on the *S_p* compared to the *R_p* isomer.

Although the titration experiments were carried out under a nitrogen atmosphere, the possibility that a slow oxidation took place could not be excluded a priori. Such an oxidation would generate normal lecithin that could be hydrolyzed by PLA₂ instead of the thiono analogue itself. Thus, the low hydrolysis rates that are given in Table II could be too high because of oxidation. Therefore, the hydrolysis was also followed by ³¹P NMR, a technique that allows the simultaneous observation of hydrolysis and of a possible oxidation. Figure 3 (panel A) shows the hydrolysis of the *R_p* isomer by native PLA₂ and the concomitant appearance of the corresponding lyso derivative. With both mutant PLA₂s similar results were obtained (data not shown). When the *S_p* isomer was incubated in the presence of the Y69F mutant, a slow hydrolysis occurred (Figure 3, panel B). From these data a specific activity of 1.6 was calculated, a value that agrees well with the activity found in the titrator (Table II). In agreement with this observation was the fact that inspection of the spectral range around 0 ppm after overnight incubation did not reveal the presence of oxidation products that could be expected to show up around this position. Overnight incubation of native PLA₂ and of Y69K mutant revealed the presence of traces of lyso derivative (data not shown) from which specific activities of less than 0.06 and 0.008, respectively, were calculated. Thus, whereas the Y69F mutant is only 2.3 times more active than native PLA₂ on the *R_p* isomer, this ratio has increased to at least 27 for the *S_p* isomer.

Activities on Neutral Phospholipid Analogues. Another modification that was made in the polar head group of the substrate molecule was the removal of the negative charge of the phosphate (Figure 1, analogue 3). This modification makes the analogue insoluble; in water it forms emulsions with the size of the droplets depending on the degree of agitation. Under these conditions no hydrolysis by PLA₂ occurs (data not shown). The triester droplets are readily soluble in diethyl ether, but in the presence of deoxycholate the system remains biphasic. It was known already that, neither in diethyl ether/buffer systems nor in the presence of deoxycholate, enzymatic hydrolysis occurred with dibenzyl esters of 1,2-

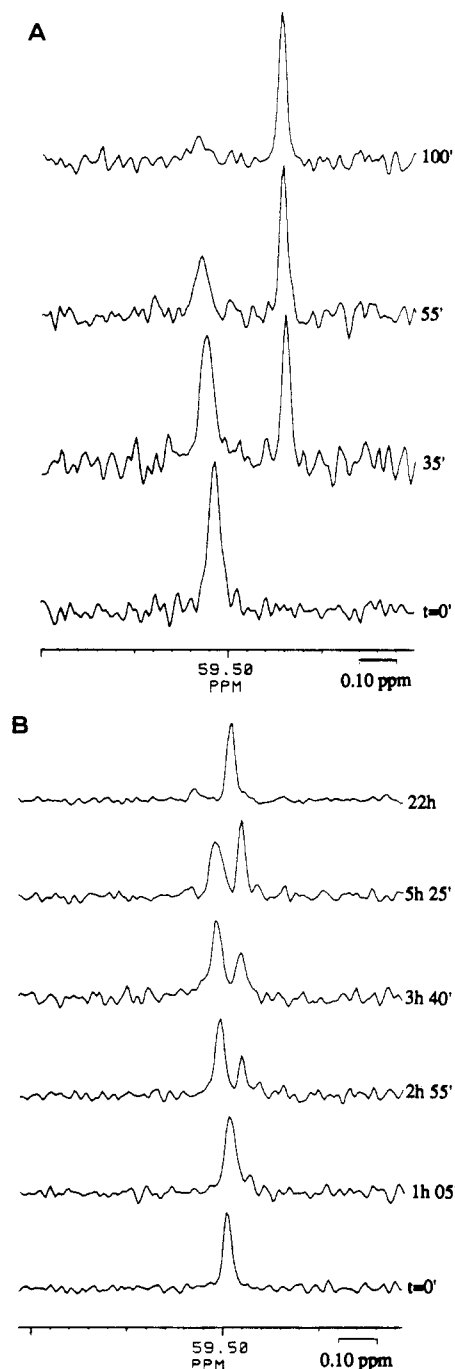


FIGURE 3: (Panel A) ^{31}P NMR spectrum of the hydrolysis of the R_p isomer of 1,2-didodecanoyl-*sn*-glycero-3-thionophosphocholine (5 mM) by wild-type PLA_2 (0.266 μM). (Panel B) ^{31}P NMR spectrum of the hydrolysis of the S_p isomer of 1,2-didodecanoyl-*sn*-glycero-3-thionophosphocholine (5 mM) by Y69F PLA_2 (0.605 μM). For experimental details, see Materials and Methods.

diacyl-*sn*-glycerol 3-phosphate and with dimethyl esters of 1,3-diacyl-*sn*-glycerol 2-phosphates (Slotboom et al., 1970). In the present study we obtained similar results with the dimethyl ester of 1,2-diacyl-*sn*-glycerol 3-phosphate. Neither in the presence of ether nor in the presence of deoxycholate or taurodeoxycholate was degradation of this substrate by PLA_2 or by the mutant enzymes observed by us (data not shown). In the presence of relatively high concentrations of the product analogue choline *n*-hexadecyl phosphate (Figure 1, analogue 4), however, the dimethyl ester gives clear solutions, probably mixed micelles, which are readily attacked by native pancreatic PLA_2 as well as by the Y69F and Y69K mutants (Table III). Again, as was seen with the thionole-

Table III: Activities of Wild-Type, Lys-69, and Phe-69 PLA_2 s on diC12PC and Its Dimethyl Ester Derivative in the Presence of the Substrate Analogue C16PN^a

enzyme	activity ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$)	
	diC12PC	diC12dimethylIPA
wild type	12	1.4
Lys-69	4.5	0.2
Phe-69	7	7

^a diC12PC, 1,2-didodecanoyl-*sn*-glycero-3-phosphocholine; diC12dimethylIPA, 1,2-didodecanoyl-*sn*-glycerol-3-(dimethyl phosphate). Assays were performed with a pH-stat, as described under Materials and Methods. Accuracy was about 10% for each given value.

cithin, the relative high activity of the Y69F phospholipase as compared to the native and Y69K enzymes on this modified substrate is worth mentioning. That this hydrolysis is due to the action of PLA_2 was shown by the following experiments. First, the reaction is calcium dependent: in the presence of EDTA no hydrolysis occurs. Second, thin-layer chromatography in ether-hexane (95:5 v/v) of the hydrolysis products of the dimethyl ester of 1-hexadecanoyl-2-tetradecanoyl-*sn*-glycerol 3-phosphate after incubation with porcine pancreatic PLA_2 shows two spots with R_f values of 0.87 and 0.26 corresponding to fatty acid and dimethyl ester of 1-acyllyso-glycerol phosphate, respectively. In this solvent system (lyso)phospholipids with a negative charge on the phosphorus remain at the origin, and hence, removal of a methyl group prior to hydrolysis can be excluded. Gas chromatographic analysis showed that in accordance with the known specificity of phospholipases A_2 exclusively myristic acid was released, whereas the lyso derivative contained palmitic acid only (data not shown).

Activities on Substrates with Modified Ester-Phosphate Distance. The next change that was made in the structure of the substrate was a modification of the distance between the phosphate and the *sn*-2 ester bond. Thus, when the phosphorus was linked to the glycerol via a direct carbon-phosphorus bond (Figure 1, analogue 5), the distance between the negative charge and the susceptible ester bond was reduced. Despite this change in the structure *rac*-1-tetradecanoyl-2-dodecanoylphosphonolecithin turned out to be a rather good substrate for phospholipase A_2 : in the presence of a 2-fold excess of taurodeoxycholate, this analogue was degraded by native PLA_2 with about 16% of the rate on normal *rac*-didodecanoyllecithin. Also the Phe-69 mutant was able to hydrolyze these substrates, but only with about 6% of the rate observed with normal *rac*-didodecanoyllecithins. In order to determine which ester bond was cleaved, we analyzed the lyso derivative and the liberated fatty acid, which were isolated after hydrolysis of the phosphonolecithin by native PLA_2 , with gas-liquid chromatography. The results indicated that the enzyme had retained full positional specificity, since only dodecanoic acid was released. To our surprise, however, we found that native porcine pancreatic PLA_2 was able to hydrolyze this racemic phospholipid to completion. Also after 100% hydrolysis the only fatty acid that was released was dodecanoic acid. Using the pure L and D isomers, we were able to quantitatively determine the activities of PLA_2 on these isomeric phosphonolipids with the pH-stat. The D isomer was hydrolyzed about 100 times more slowly than the L isomer. Although the rate of hydrolysis of the D isomer is quite low, the difference with nonenzymatic hydrolysis is large. Qualitatively, this is illustrated by the fact that in racemic mixtures of phosphonolipids the phosphonolipid is hydrolyzed 100% by native and the two mutant PLA_2 s. In contrast, racemic mixtures of normal phospholipids were degraded 50% only by

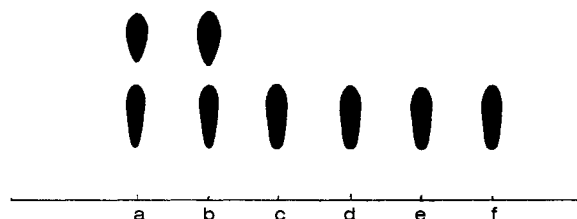


FIGURE 4: Degradation of *rac*-1,2-didodecanoyllecithins and *rac*-1-myristoyl-2-dodecanoylphosphonolecithins by wild-type, Y69K, and Y69F PLA₂s. Incubations were performed in the presence of sodium taurodeoxycholate (2 mol/mol of phospholipid) in 50 mM borate buffer containing 5 mM CaCl₂ at pH 8.0 in a total volume of 500 μ L for 8 h at 37 $^{\circ}$ C. Lanes a–c show the degradation products of *rac*-1,2 didodecanoyllecithins by wild-type (25 μ g), Y69K (100 μ g), and Y69F (100 μ g) PLA₂s, respectively. Lanes d–f show the degradation products of *rac*-1-myristoyl-2-dodecanoylphosphonolecithins by wild-type (25 μ g), Y69K (100 μ g), and Y69F (100 μ g) PLA₂s, respectively. The TLC plate was developed in CHCl₃–MeOH–H₂O (65:35:8 by volume), and spots were visualized by spraying with phosphorus reagent. The upper spots represent nondegraded lecithin or phosphonolecithin, whereas the lower spots represent the lyso derivatives of these compounds.

native and the Y69K mutant but 100% by the Y69F mutant (Figure 4). From these data we conclude that, with the phosphonolecithin as a substrate, the enzyme retains its positional specificity but loses part of its stereospecificity.

Increasing the distance between the phosphate and the ester bonds as in *rac*-1-hexadecanoyl-2-tetradecanoyl-1,2,4-butanetriol phosphate (Figure 1, analogue 6) completely abolished the activity of both native and the mutant phospholipases.

DISCUSSION

From results obtained with phospholipases in which Tyr-69 was nitrated, Meijer et al. (1979) concluded that the side chain of this residue is involved in the interaction of PLA₂ with monomeric and with micellar substrate analogues. Our results support this conclusion for the monomers, but the role of Tyr-69 in the interaction with micelles is less clear. Addition of monomeric choline phosphate *n*-dodecyl to the Y69F mutant does not give rise to the spectral changes, characteristic of tyrosine perturbations (Donovan, 1969), that are observed with the native PLA₂. The small signal that is observed with the Y69F PLA₂ has the characteristics of a tryptophan perturbation, and this signal might very well be hidden in the spectrum of the native PLA₂ by the large peak at 289 nm. The fact that a tryptophan perturbation is observed in the ultraviolet spectra agrees with the appearance of fluorescence signals upon addition of monomeric concentrations of choline *n*-dodecyl phosphate. Weak signals are obtained with the Y69K mutant (Figure 2). This might be explained by the small distance between the side chains of Lys-69 and Trp-3. Although the α -carbon atoms are about 12 \AA apart, the charged ϵ -NH₃⁺ group and the indole ring may approach each other to less than a few angstroms. This distance is small enough to cause quenching of the fluorescence, and it might change the environment of the tryptophan enough to prevent large spectral changes once a monomeric lipid molecule is bound to the active site. The addition of micellar concentrations of choline phosphate *n*-hexadecyl to native and Y69F PLA₂ gives rise to spectra that are dominated by a tryptophan signal. Again, no significant signals were obtained with the Y69K mutant, which suggested once more that in this mutant Trp-3 is influenced by the side chain of Lys-69. A similar sensitivity of Trp to small changes in the amino acid sequence has been demonstrated also for native *Crotalus adamanteus* and *Crotalus atrox* venom PLA₂s. These PLA₂s contain three Trp residues at positions 21, 31 and 117 (homology num-

bering), differ by only six other substitutions (Verheij et al., 1981a), and as all PLA₂s require calcium ions for activity. Upon addition of calcium ions the *C. adamanteus* enzyme shows large spectral perturbations, which have been ascribed to the removal of a charged group from the vicinity of a Trp residue (Wells, 1973). Addition of calcium ions to the *C. atrox* enzyme does not produce spectral changes (Purdon et al., 1977). The quenching of lipid-induced spectra in native PLA₂ by Lys-69 is not a general phenomenon. Native *C. atrox* PLA₂, which contains a lysyl residue at position 69, produces large spectral changes in the presence of choline *n*-hexadecyl phosphate [data not shown; see also Verheij et al. (1981b)]. The fact that this venom PLA₂ contains three Trp residues at positions 21, 31, and 117 but no aromatic residue at position 3 makes it difficult to draw definite conclusions from these experiments.

From the spectral data no quantitative affinities of the Y69K mutant for micelles can be obtained. Qualitatively, the use of gel filtration showed that the Y69K mutant has affinity for micelles of choline *n*-hexadecyl phosphate. This conclusion is supported by the kinetic data of native and mutant PLA₂s (Table I) with several aggregated substrates, since without binding to these aggregates one would not observe activity. The K_m^{app} values that were obtained with dioctanoyllecithin show that no large changes have occurred in the affinity for aggregated substrate molecules as a result of the mutation of Tyr-69 into phenylalanine or lysine. Meijer et al. (1979) based their conclusions about the role of Tyr-69 in the interaction with aggregated lipids on nitrated and on dansylated PLA₂. The fact that nitrated tyrosine is a stronger acid than tyrosine implies that at pH 8 nitrotyrosine carries a negative charge. It has been discussed before (Verheij et al., 1981a) that a negative charge at the lipid binding domain interferes with binding. On the other hand, the introduction of a large hydrophobic group like the dansyl moiety obviously improves hydrophobic interactions.

In the 56 phospholipases A₂ that have been sequenced at present (van den Bergh et al., 1989b), tyrosine and lysine are found with about equal frequency at position 69, although in pancreatic enzymes invariably a tyrosine is present. In active PLA₂s only once was a different residue, i.e., a phenylalanine, reported in the enzyme from *Laticauda colubrina* venom (Takasaki et al., 1988). Given the observation that in naturally occurring PLA₂s lysine and tyrosine both can be present at this position, the low activity of the Y69K mutant relative to native porcine pancreatic PLA₂ was unexpected. It must be realized, however, that Tyr-69 is adjacent to a surface loop of variable conformation in pancreatic enzymes, whereas this loop is absent in all venom PLA₂s. It has been shown recently (Kuipers et al., 1989b) that deletion of this loop in pancreatic PLA₂ markedly increases activity on short-chain lecithins. The greater flexibility of a lysyl side chain compared to that of tyrosine in combination with the presence of the mobile surface loop might explain the low catalytic power of the Y69K pancreatic PLA₂ mutant.

For many years phospholipase A₂ has been regarded as an enzyme that degrades phospholipids in a calcium-dependent reaction with high stereospecificity and positional specificity. In a recent paper (Kuipers et al., 1989a) it was shown that replacement of Tyr-69 by a phenylalanine resulted in a partial loss of the stereospecificity. In the present study we also included the substitution of Tyr-69 by Lys, and the results obtained with this mutant confirm our previous notion that the stereospecificity at *sn*-2 of the glycerol backbone is dependent on the presence of a hydrogen bridge donor in the side

chain of residue 69 of porcine pancreatic PLA₂. Also, the fact that Y69K PLA₂, like native PLA₂, only degrades the R_p isomer of thionolecithins while the Y69F mutant can degrade both R_p and S_p isomers (Table II) strongly suggests that it is indeed the hydrogen bridge from either Tyr-69 or Lys-69 to phosphate that determines to a large extent stereospecificity both at *sn*-2 and at phosphorus. Because the Y69F mutant is able to degrade both D and L phospholipids, detailed kinetic analyses of racemic compounds like dihexanoyldithionolecithin (Table I) is strictly impossible. However, because the activity of the Y69F mutant on D-lecithins is about 50 times lower than that on L-lecithins (Kuipers et al., 1989), the resulting error is small compared to standard deviations caused by the test system itself. The same reasoning applies to the hydrolysis of the racemic phosphonolecithin by native and mutant phospholipases.

The activity of native and Y69K pancreatic PLA₂s on 1,2-didodecanoylthionolecithin is about 4-fold lower than that on the regular 1,2-didodecanoyllecithin. This reduction in activity is low compared to the 24-fold reduction reported by Tsai et al. (1985) for the bee venom PLA₂-catalyzed hydrolysis of 1,2-dihexadecanoylthionolecithin. This difference might be explained by the detergent used: taurodeoxycholate in this study and Triton X-100 by Tsai and co-workers, and/or by the different fatty acyl chain lengths. Another explanation could be that bee venom phospholipase A₂, despite its functional homology with other extracellular PLA₂s, might be structurally distinct in its phosphate binding pocket. The structural homology of bee venom phospholipases and pancreatic or snake venom phospholipases has indeed been questioned before (Verheij et al., 1981a; Maraganore et al., 1987). That substitutions at the entrance to the active site can change the preference of a given PLA₂ from regular phospholipids to thiono analogues is illustrated by the Y69F mutant which, in contrast to native PLA₂, prefers thionophospholipids to regular ones. Since this mutant, in contrast to native and Y69K PLA₂s, also has measurable activity on the S_p isomer, we conclude that the hydrogen bridge that can be formed between phosphate and Tyr-69 or Lys-69 is of major importance for stereospecificity at phosphorus. A question that remains to be answered is whether this hydrogen bridge occurs between the amino acyl side chain and the P oxygen or the P sulfur when the R_p isomer is bound to the active site. Several arguments seem to support an interaction with the sulfur. Due to the hydrogen-bonding capacities and the size, a substitution of sulfur for oxygen would reduce the interaction with Tyr-69 or Lys-69 on account of a weaker hydrogen bond and for steric reasons. Thus the introduction of a sulfur on phosphorus would make the thiono analogue a weaker substrate than the parent lecithin. In the mutant Y69F no such effects are evident, and the fit with sulfur could even be favored by hydrophobic interactions. Another argument that fits the proposed orientation of the sulfur is the observation by Tsai et al. (1985) that replacement of calcium by cadmium ions causes to a large extent the reversal in the preference of the enzyme for the S_p and R_p isomers. Given the preference of cadmium for sulfur over oxygen as a ligand, this also supports an orientation with the sulfur in the R_p isomer in proximity of Tyr-69 or Lys-69.

As early as 1968 it was shown (de Haas et al., 1968) that the presence of a negative charge on phosphorus is essential for phospholipase A₂ activity in the presence of deoxycholate or diethyl ester. The results presented in the present study show that this conclusion has to be revised to some extent. Phosphorus triesters like didodecanoylphosphatidic acid di-

methyl ester are practically insoluble in aqueous buffers and are hence not degraded by PLA₂. Addition of diethyl ether, yielding two clear phases, does not promote hydrolysis, probably because the triester is hardly surface active and, therefore, does not accumulate at the ether-water interface. Deoxycholate apparently is not readily incorporated into the triester droplets, and formation of mixed micelles does not seem to occur readily: even at a 10-fold molar excess triester droplets are still visible, and hydrolysis is negligible. In the presence of the product analogue choline *n*-hexadecyl phosphate, clear solutions of presumably mixed micelles are formed. In these micelles the triester is degraded at significant rates compared to lecithin although the latter is degraded more slowly in this detergent than in the presence of deoxycholate (compare Tables II and III). Since choline *n*-alkyl phosphates are product analogues and hence probably competitive inhibitors, these low hydrolysis rates were not unexpected. More striking is the fact that the two mutant PLA₂s behave quite differently. Whereas the Y69K mutant has the same relative preference as native PLA₂ for lecithin over triester, the Y69F mutant has equal activities on both surfaces. An attractive explanation is that upon introduction of two methyl groups in the phosphate moiety the interaction with the hydrogen-bond donors Tyr and Lys is weakened, whereas the methyl group(s) still can have a hydrophobic interaction with Phe. In all cases the Ca²⁺ ion could still be liganded by the one free oxygen atom at the phosphorus. As a result of both interactions the latter mutant can still have a rather high affinity for the triester. Unfortunately, direct verification of this affinity is experimentally difficult because of the low solubility of the triester in water. Estimations of this affinity after codispersion of the triester in micelles is impossible, since under these conditions several equilibria are involved (Verger & de Haas, 1976).

The finding that the phosphonolecithin (Figure 1, analogue 5) is a substrate, while the butanetriol lecithin (Figure 1, analogue 6) is not degraded at all, was not completely unexpected. It has been reported by Bensen et al. (1972b) that the dihexanoyl- and dioctanoylphosphonolecithins are degraded with 15% and 5%, respectively, of the rates observed with the corresponding phospholipids. The same authors reported that the dihexanoylbutanetriol analogue was completely resistant to the action of PLA₂. Our results with the phosphonolecithin show that this compound is hydrolyzed by PLA₂ with retention of the positional specificity but with a loss of the stereospecificity of the enzyme. This furthermore suggests that the results that were obtained by Bensen et al. (1972b) with the short-chain derivatives were not caused by the aspecific hydrolysis of loosely bound substrate.

Because the phosphonolecithin analogue is degraded more slowly than the normal lecithin, the question arises whether this reduction in hydrolysis rate is caused by an improper orientation of either the *sn*-2 ester bond or of the phosphonate in the phosphonolecithin. If the susceptible ester bond had been shifted over a significant distance in the active site, a large effect on the rate of catalysis would have been expected. Since the reduction in the rate of hydrolysis is only moderate and because the enzyme retains its positional specificity, we presume that, upon binding of the phosphono substrate in the active site, the acyl ester bond remains in place. Consequently, we also presume that, as a result of the reduced distance between the acyl ester bond and the phosphonate moiety in the phosphono substrate, this phosphonate occupies a somewhat different position in the active site than the phosphate of normal phospholipids. For this reason it is not clear whether

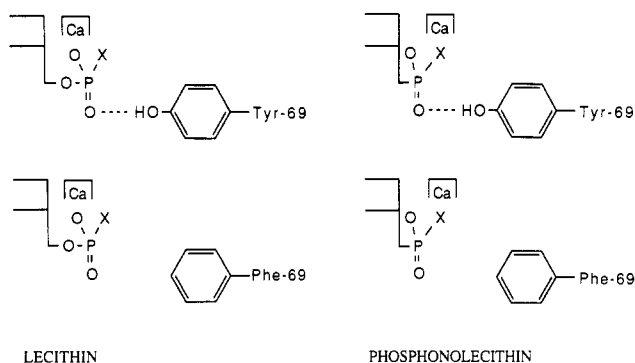


FIGURE 5: Schematic representation of the proposed relative positions of the Ca^{2+} ion, residue 69 of wild-type and mutant PLA_2 , and the phosphate moiety of lecithin and phosphonolecithin: (Panel A) interactions of lecithin with wild-type PLA_2 ; (panel B) interaction of lecithin with Phe-69 mutant PLA_2 . (Panels C and D) These interactions with a phosphonolipid. The charges of the Ca^{2+} ion and of the phosphate are not indicated for reasons of clarity.

an interaction of the phosphonate can still exist with Ca^{2+} and/or with the side chain of Tyr-69.

In considering the possibility of an interaction between the phosphonate and the Ca^{2+} ion, one has to keep in mind that the Ca^{2+} ion is relatively small and is kept in a fixed position by the side chain of Asp-49 and the backbone carbonyl oxygens of residues Tyr-28, Gly-30, and Gly-32. Therefore, we assume that it is highly improbable that the Ca^{2+} ion is able to shift even over a small distance in the enzyme, and we consider an interaction between phosphonate and the Ca^{2+} ion in phosphonolipids improbable. As to the possibility of an interaction between Tyr-69 and the phosphonate moiety of the phosphonolipid, it should be noticed that rotation of the side chain around the α -carbon atom can change the position of the phenolic hydroxyl group considerably. A displacement as large as 12 Å has indeed been reported for Tyr-248 in carboxypeptidase A, to accommodate the binding of a substrate molecule (Rees & Lipscomb, 1982). Also for bovine PLA_2 a shift of the position of Tyr-69 has been reported. This residue occupies an exposed position in native PLA_2 , but it moves inside the substrate binding pocket in a PLA_2 modified by the introduction of a *p*-bromophenacyl group, covalently attached to the active-site histidine (Renetseder et al., 1988). These two observations are indicative of a high mobility of surface tyrosine residues in general and of Tyr-69 in PLA_2 in particular. With phospholipids, it was shown that a hydrogen bond between Tyr-69 and phosphate is crucial for stereospecificity, which suggests that Tyr-69 indeed shifts toward the inside of the enzyme upon binding of a substrate molecule. In analogy, we assume that Tyr-69, by shifting even further toward the inside of the enzyme, is still able to interact with the phosphonate moiety of a phosphonolipid. In Figure 5 a schematic representation of the orientation of a phospholipid and of a phosphonolipid in the active site of PLA_2 is given. Although direct proof for the proposed hydrogen bond between Tyr-69 and the phosphonate of phosphonolipids is lacking, there are some kinetic data which are consistent with such an interaction. When Tyr-69 was replaced by Phe, the activity of the enzyme was reduced about 3-fold, not only the phospholipids but also with phosphonolipids as substrate. This suggests that the mutation Tyr-69 to Phe prevents a hydrogen bond with both the phosphate and the phosphonate. For the Y69F mutant acting on glycerophospholipids and for native phospholipase acting on phosphonolipids, the effects on stereospecificity are qualitatively and quantitatively similar. Thus it seems that a sole interaction either between calcium and phosphate (Phe-69 PLA_2 and lecithin) or between Tyr-69 and

phosphonate (wild-type PLA_2 and phosphonolecithin) is not enough for the maintenance of stereospecificity.

Considering the function of the Ca^{2+} ion in PLA_2 , in 1980 a double role for the Ca^{2+} ion was proposed (Verheij et al., 1980). First, Ca^{2+} was supposed to bind the negative charge of the phosphate, and second, Ca^{2+} was supposed to enhance the polarization of the carbonyl oxygen of the ester at the 2-position. Our results underline and extend this previous notion, by showing that Ca^{2+} and Tyr-69 together can fix the position of the phosphate moiety of phospholipids, thereby securing stereospecificity. With some substrates (phosphonolecithin, phosphatidic acid dimethyl ester), where a strong interaction of Ca^{2+} with phosphate is less obvious, the Ca^{2+} ion is still indispensable for enzymatic activity. This observation is in agreement with the proposed catalytic role of Ca^{2+} . Whether this role is a direct one, such as the polarization of the carbonyl oxygen of the 2-acyl ester bond, or an indirect one like the stabilization of the active conformation of PLA_2 , suggested by Tsai et al. (1985), remains a subject of further studies.

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Steady-State and Laser Flash Induced Photoreduction of Yeast Glutathione Reductase by 5-Deazariboflavin and by a Viologen Analogue: Stabilization of Flavin Adenine Dinucleotide Semiquinone Species by Complexation[†]

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ABSTRACT: Steady-state and laser flash photolysis techniques have been used to examine the photoreduction of yeast glutathione reductase by the one-electron reduction products of 5-deazariboflavin and the viologen analogue 1,1'-propylene-2,2'-bipyridyl. Steady-state photoreduction of the enzyme with the viologen generates the two-electron-reduced form, whereas photoreduction with deazaflavin generates the anion semiquinone. Flash photolysis indicates that the product of viologen radical reduction is also a semiquinone, suggesting that this species is rapidly further reduced by viologen in the steady-state experiment to form the EH₂ enzyme. This reduction is apparently inhibited when deazaflavin is the photoreductant, perhaps due to complexation of the anion semiquinone with deazaflavin. Steady-state experiments demonstrate that complexation of the anion semiquinone with NADP⁺ also inhibits further reduction. Both one-electron reduction reactions of oxidized glutathione reductase proceed at close to diffusion-controlled rates (second-order rate constants = 10⁸-10⁹ M⁻¹ s⁻¹), despite the relatively buried nature of the FAD cofactor. Addition of NADP⁺ and oxidized glutathione produced no effects on the kinetics of the initial entry of the electron into the enzyme. No kinetic evidence of intramolecular electron transfer involving the FAD and the protein disulfide was obtained during or subsequent to the initial one-electron reduction process. Thus, if this reaction occurs in the semiquinone, it must be quite rapid ($k > 8000$ s⁻¹).

Glutathione reductase (GR) catalyzes the reduction of the disulfide bond of oxidized glutathione (GSSG), using NADPH as the source of reducing equivalents and an FAD cofactor and a protein disulfide as intermediate electron carriers

(Williams, 1976). The enzyme isolated from human erythrocytes has been extensively characterized, and a refined X-ray structure at 1.54-Å resolution has been reported (Karplus & Schultz, 1987a). It is a dimer (total MW = 105 000) consisting of two identical subunits, each containing one FAD molecule (Krauth-Siegel et al., 1982). In the first part of the catalytic reaction, the enzyme is reduced to its stable EH₂ form by NADPH, from which NADP⁺ dissociates. This species has an open disulfide (Cys-58-Cys-63 in the human enzyme; Pai & Schulz, 1983) resulting from electron transfer via the flavin, and spectroscopic data indicate the existence of a charge-transfer complex between a thiolate anion (the proximal

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